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Factors governing single-trial contextual fear conditioning in the weanling rat

M.A. Burman, N.J. Murawski, F.L. Schiffino, J.B. Rosen, and M.E. Stanton

Abstract

Although contextual fear conditioning emerges later in development than explicit-cue fear conditioning, little is known about the stimulus parameters and biological substrates required at early ages. The current experiments adapted methods for investigating hippocampus function in adult rodents to identify determinants of contextual fear conditioning in developing rats. Experiment 1 examined the duration of exposure required by weanling rats at postnatal day (PND) 23 to demonstrate contextual fear conditioning. This experiment demonstrated that 30 s of context exposure is sufficient to support conditioning. Furthermore, preexposure enhanced conditioning to an immediate footshock, the *context preexposure facilitation effect* (CPFE), but had no effect on contextual conditioning to a delayed shock. Experiment 2 demonstrated that NMDA receptor inactivation during preexposure impairs contextual learning at PND 23. Thus, the conjunctive representations underlying the CPFE are NMDA-dependent as early as PND23 in the rat.

Keywords

Context; Fear; Development; NMDA receptor; MK-801

Contextual fear conditioning requires rodents to associate a fear-inducing unconditioned stimulus (US; e.g. a footshock) with a representation of the experimental apparatus in which the shock occurs (the context). In addition to the amygdala, which is required for fear conditioning in general (Davis, 2006; Fanselow & LeDoux, 1999; LeDoux, 2003), contextual fear conditioning requires additional neural structures such as the hippocampus (Kim & Fanselow, 1992; Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992). Interestingly, not only does contextual fear conditioning require different neural substrates, but it also has a different ontogenetic profile than explicit-cue fear conditioning (Rudy, 1993; Rudy & Morledge, 1994; Stanton, 2000). Although cued-fear conditioning emerges prior to postnatal day (PND) 18, contextual fear conditioning does not emerge until PND 23.

Much is already known about the ability of young rats to undergo contextual fear conditioning. Rudy and colleagues have demonstrated that 18-day-old rats show equivalent

Address correspondence to either: Mark Stanton, Department of Psychology, University of Delaware, Newark, DE 19716, stanton@psych.udel.edu. Or Mike Burman, Department of Psychology, Bates College, Lewiston, ME 04240. mburman@bates.edu.

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post-shock freezing as 23- and 32-day-old rats. However, 18-day-old rats show much less long-term retention of contextual fear, suggesting that they are unable to form a long-term memory of the conditioning episode (Rudy & Morledge, 1994). Further research has demonstrated that the ability to show contextual fear conditioning emerges rapidly between PND 18 and 23 (Rudy, 1993; Stanton, 2000).

Despite this progress, there is still much we do not know regarding contextual fear conditioning in developing rats. In adult animals, there is a linear relationship between amount of exposure (the placement-to-shock interval) and contextual fear conditioning such that animals exposed to an aversive stimulus with little or no previous exposure to the context show very little subsequent freezing (termed the immediate shock deficit) and levels of freezing increase as the duration of prior exposure increases (Fanselow, 1990; Frankland et al., 2004; Wiltgen, Sanders, Behne, & Fanselow, 2001). If the hippocampus were still immature (see Rudy, 1992; Stanton, 2000), weanling rats may require additional exposure to form a contextual representation and thus show context conditioning. Experiment 1 therefore extended to weanling rats the observation that the formation of the contextual representation can be separated from the fear learning component of the task by providing context exposure on one day and a shock immediately upon placement into the context on another day (Fanselow, 1990; Frankland et al., 2004; Lattal & Abel, 2001; Matus-Amat, Higgins, Sprunger, Wright-Hardesty, & Rudy, 2007; Rudy, Barrientos, & O'Reilly, 2002; Rudy & Morledge, 1994). Furthermore, although it is often assumed that contextual fear conditioning at PND 23 requires the same neural mechanisms as fear conditioning in the adult rat (Rudy, 1992, 1993; Stanton, 2000), the biological substrates of contextual fear conditioning around the age when task performance emerges have yet to be investigated. The question is important in light of evidence that context conditioning can be supported by “elemental associations” that do not depend on the hippocampus (Rudy & O'Reilly, 2001), especially during the weanling period (Pugh & Rudy, 1996). Therefore, Experiment 2 confirmed that NMDA receptor activation during preexposure to the context at PND 23 is required for the CPFE, as is the case in adult rats (Stote & Fanselow, 2004).

Experiment 1

This experiment is the first to systematically vary the amount of contextual exposure prior to a single footshock in weanling rodents. As PND 23 is the first day in which contextual fear conditioning has been demonstrated, we asked whether weanling rats would require longer placement-to-shock intervals to show contextual fear conditioning than is typically reported in adults and whether contextual preexposure would enhance contextual fear conditioning at various placement-to-shock intervals.

Methods

Subjects—A total of 130 Long Evans weanling rats (64 male, 66 female) that were the offspring of 20 different mothers were used in this study. Rats were bred in the University of Delaware Animal Facility from breeders derived from Harlan Long Evans stock. All experimental procedures were approved by the University of Delaware Internal Animal Care and Use Committee (IACUC). Litters were weighed and culled to eight pups (4 male, 4 female when possible) on PND 3, and were left undisturbed until weaning on PND 21.

Dams were housed with their litters in clear Polypropylene containers measuring 8" high×18" long×9" wide in an animal facility that was operated according to NIH guidelines. The housing facility was maintained on a 12:12 hour light/dark cycle with lights on at 7 a.m. The age of litters was determined by daily checks during the light part of the cycle with gestational day 22 defined as the day of birth (PND 0). After weaning from their mothers on PND 21, pups were housed with same sex littermates and continuously supplied with food and water except during experiments. No more than a single same-sex littermate was assigned to a given experimental group.

Apparatus and stimuli—Fear conditioning occurred in 4 conditioning chambers (11 × 17.5 × 16 cm) placed within a fume hood. The chambers were made of clear plastic, except for sides facing another conditioning chamber, which were made opaque. The bottom of each cage was a grid floor (11.5 cm from top of chamber) through which footshocks were delivered. The grid bars were 0.5 cm in diameter and placed 1.25 cm apart. The US was a 1.5-mA, 2-s, footshock delivered by a shock scrambler (Med Associates, Georgia, VT ENV-414S). Movement was recorded and immobility assessed using FreezeFrame software (Actimetrics, Wilmette IL) set to the 4 chamber/1 camera mode.

The other preexposure context consisted of a completely different set of chambers in a different part of the building. These chambers were 22×22×26 cm wire mesh cages enclosed in larger sound-attenuated chambers (BRS/LVE, Laurel, MD) lined with sound-absorbing foam.

Behavioral Procedure—Context preexposure occurred on PND 23, training on PND 24 and testing on PND 25. All sessions began between 2:00 and 4:00 pm. Prior to each session, rats were weighed and placed into individual transport cages. The transport cages were 24×18×13 cm and made of white plastic. They were covered with a wire top and a sheet of white paper placed on top to render all sides opaque. Cohorts were no larger than 16 animals.

For preexposure, rats were run in groups of 2–4 and placed for 5 minutes into either the footshock chamber where they were to be conditioned the next day or a completely separate context to control for handling and exposure to novelty. For preexposed-other rats, we controlled for handling and transport by placing them in the different context (see above). In this case, the transport cages were not covered with the white paper, to provide a somewhat different transport experience.

For training, rats were run in groups of 2–4 and placed into the chambers approximately 30 s, 60 s, 120 s or 300 s prior to the delivery of the single footshock. Additional groups received a shock immediately upon placement into the chamber (within 5 s) or received 300 s of exposure without footshock. Rats were loaded into the chambers 2 at a time and thus the first pair of rats loaded received slightly more exposure (approx 5–10 s). To ensure that the shock was delivered quickly, immediate shock rats were run only 2 at a time. Subjects were removed from the chambers as quickly as possible following training in the same order in which they were loaded, and left in their transport cage until all rats had finished the

experiment for the day. At this point the rats were returned to group housing and left for approximately 24 hours until testing.

For testing, rats were run in identical circumstances as for training except that the testing session lasted for 300 s for all animals and no shock was delivered.

Data analysis and Statistics—The data were analyzed using FreezeFrame software (Actimetrics, Wilmette IL). The bout length was set at 0.75 s and the freezing threshold (change in pixels/frame) was initially set as described in the instructions. A human observer verified the setting by watching the session and adjusting the threshold if necessary to ensure that small movements were not recorded as freezing. Once percent freezing was determined, the data were imported into Statistica 7 data analysis software.

One female rat was dropped from the experiment because of procedural error. The rats that remained were assigned as follows: 6 female and 6 male rats to the preexposed no shock group, 6 female and 6 male rats to the preexposed-other no shock group, 7 female and 7 male to the preexposed immediate shock group, 7 female and 7 male to the preexposed-other immediate shock group, 6 female and 7 male rats to the preexposed 30-s placement-to-shock group, 7 female and 7 male to the preexposed-other 30-s placement-to-shock group, 6 female and 6 male rats to the preexposed 120 s placement-to-shock group, 8 female and 6 male to the preexposed-other 120 s placement-to-shock group, 6 female and 6 male to the preexposed 300 s placement-to-shock group, and 5 female and 6 male to the preexposed-other 300 s placement-to-shock group. The data were collected and analyzed in a $2 \times 2 \times 5$ factorial ANOVA (with sex, preexposure condition and placement-to-shock as independent factors). Newman-Keuls post-hoc tests were used to further examine trends found in the ANOVA.

Results and Discussion

Preexposure ameliorated the immediate shock deficit but otherwise had no effect on conditioning (see Figure 1). The enhancement of contextual fear conditioning to an immediate shock by preexposure (CPFE) is consistent with previous findings in weanling (Rudy and Moreledge, 1994) and adult (e.g. Fanselow 1990) rats. There was no effect of gender ($F(1,109) = 0.11, p > .10$), nor any interactions with gender ($ps > .10$). There was no main effect of preexposure, ($F(1,109) = 0.90, p > .10$), suggesting that preexposure did not generally enhance conditioning. The effects of preexposure in adult rodents have been found to be somewhat inconsistent as preexposure sometimes enhances contextual fear generally (Frankland et al., 2004) and other times only enhances conditioning at a subset of placement-to-shock intervals (Fanselow, 1990). There was a main effect of placement-to-shock ($F(4,109) = 13.25, p < .01$), representing the lower levels of conditioning in the no shock and immediate shock conditions. There was also a preexposure $\times$ placement-to-shock interaction ($F(4,109) = 4.07, p < .01$), reflecting the enhanced conditioning caused by preexposure in the immediate shock group but not the other groups. Post-hoc Newman-Keuls tests show that the no shock groups did not differ from the preexposed-other immediate shock group, but that these three groups differed significantly from every other group ($ps < .05$), with the exception of the preexposed 300s placement-to-shock group, which

only trended towards a significant difference from the no shock groups ($p < .10$). Slightly lower conditioning following 5 minutes of context exposure has been previously observed in adult mice (Frankland et al., 2004).

Experiment 2

Experiment 2 extended the previous results by beginning to investigate the biological substrates of contextual conditioning in weanling rodents. Experiment 1 demonstrated that preexposure on PND 23 was sufficient for animals to form a representation of the context to facilitate conditioning to an immediate shock on PND 24. Experiment 2 asked whether NMDA-type glutamate receptor activation during preexposure is necessary for preexposure to facilitate conditioning to an immediate footshock (CPFE).

Methods

Subjects—A total of 55 Long Evans weanling rats (24 male, 31 female) derived from 8 different litters were used in this study. The details of housing and husbandry were exactly as above. Again, no more than a single same-sex littermate was assigned to a given experimental group, except in one inadvertent case where the data were averaged and treated as a single observation.

Apparatus and stimuli—The equipment and stimuli were identical to those above.

Behavioral Procedure—Preexposure, training and testing were conducted identically to Experiment 1, except that only the immediate shock and 120-s placement-to-shock interval groups were used. Furthermore, 30 minutes (± 5 min) prior to preexposure, weanling rats were injected with either 1 ml/kg 0.09% sterile saline or 1 ml/kg of 0.1 mg/ml MK-801 solution. This dose was chosen based upon previous work in weanling rats on spatial discrimination reversal learning in the T-maze (Chadman, Watson, & Stanton, 2006). All rats were then placed for 5 minutes into the footshock chamber where they were to be conditioned the next day.

Data Analysis and Statistics—One rat was excluded from the experiment for procedural error. This left 14 rats in the MK-801 120-s shock condition (7 male and 7 female), 12 rats in the saline 120-s shock condition (8 female and 4 male), 14 rats in the MK-801 immediate shock condition (8 female and 6 male), and 13 rats in the saline immediate shock condition (7 male and 6 female).

An independent sample t-test was performed on freezing levels during the preexposure session to examine whether MK-801 had any effect on baseline freezing levels. Our hypothesis for this experiment was that the incidental learning that occurs during context preexposure would be NMDA-receptor dependent. We therefore predicted that animals injected with MK-801 during preexposure would perform similarly to the preexposed-other condition from Experiment 2, whereas animals injected with saline would perform similarly to the preexposed-same condition. Thus, MK-801 during preexposure should impair only the animals shocked immediately upon placement into the chambers and not those shocked 2 minutes after placement. Due to the strong nature of our hypothesis, we used planned

comparisons to analyze the data. A 2×2×2 factorial ANOVA was also run (with sex, treatment and placement-to-shock as independent factors).

Results and Discussion

NMDA receptor activation is required during context preexposure in order for it to facilitate later conditioning to an immediate footshock (see Figure 2). Consistent with expectations, there were no main effects of sex ($F(1,45) = 1.58, p > .10$) or MK-801 treatment ($F(1,45) = 1.57, p > .10$). There was a main effect of placement-to-shock ($F(1,45) = 7.74, p < .01$), representing weaker conditioning in the immediate shock group across treatments. There was a trend towards an interaction between treatment and placement-to-shock ($F(1,45) = 3.06, p < .09$). No other interactions were significant ($ps > .10$). The planned comparison, based upon our expectation that MK-801 would impair immediate-shock conditioning but not delayed shock conditioning, confirmed our hypothesis. In the immediate shock condition, rats receiving MK-801 during context preexposure were significantly impaired compared to their saline counterparts ($p < .05$), indicating that MK-801 impaired the incidental learning that normally occurs during context preexposure. This was not the case for rats in the 120-s placement-to-shock condition ($p > .10$). This provides evidence that general contextual and shock processing, as well as motor function, on training and test days were not affected by enduring effects of MK-801 administration during preexposure.

Levels of freezing were generally low during preexposure ($3.47 \pm 0.59\%$ for saline vs. $1.45 \pm 0.71\%$ for MK-801) making it highly unlikely that differences in initial movement levels could account for a difference in the ability of rats to form a representation of the context. However, this difference did yield a significant reduction in freezing levels in MK-801 as compared to saline animals ($t = 2.24, p < .05$). This suggests that MK-801 may have caused motoric activation or hyperactivity resulting in greater movement. Since MK-801 treated animals exposed to a footshock following 2 minutes of context exposure showed intact conditioning, the deficit seen in the immediate shock group on test day cannot be accounted for by lingering effects of MK-801 on motor activity.

General Discussion

The current experiments examined behavioral and biological factors governing single-trial contextual fear conditioning around the age when task performance first develops. Rats given an immediate shock failed to show conditioning whereas delaying shock for 30 seconds supported conditioning and there was no effect of manipulating the placement-to-shock interval beyond 30 s. Thus, PND 23 rats do not appear to require greater exposure than is typically reported in adult rats to form a representation of the context (Fanselow, 1990; Frankland et al., 2004; Wiltgen et al., 2001). To ensure that the lack of difference at placement-to-shock interval beyond 30 s was not caused by a ceiling effect in the amount of freezing able to be expressed by weanling rats in our apparatus, additional rats were first subjected to the original procedure and then given an additional 3 training trials 24 hours following the initial test session (unpublished observations). When freezing was reassessed in these animals, percent freezing had increased to over 70%. These levels are consistent with those observed with fear conditioning to a tone CS at these ages (Stanton, 2000). Thus,

it appears that a ceiling in the amount of freezing weanling rats are capable of cannot account for the lack of a gradient in the levels of contextual fear observed. Rather, it appears that once a contextual representation is formed in weanling rats, additional exposure time provides no further benefit.

Several researchers have suggested that the age of emergence of contextual fear conditioning (PND 23) can be used as probe for assessing hippocampus development (Rudy, 1993; Rudy & Morledge, 1994) or the interaction between the hippocampus and amygdala (Stanton, 2000), due to the well-established role for the hippocampus in contextual fear conditioning in adult rats (Kim & Fanselow, 1992; Phillips & LeDoux, 1992), but see (Gewirtz et al., 2000). Indeed, as has been noted, the specific contextual fear conditioning deficit reported in younger animals compared with relatively intact explicit-cue fear conditioning (Rudy, 1993; Stanton, 2000), suggests that it is not the motor or sensory systems but rather it is neural structures involved in learning about the context that are late to develop.

Lesion work in adult animals suggests that rats can use either a hippocampus-dependent strategy or a hippocampus-independent strategy to demonstrate contextual freezing (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Gewirtz, McNish, & Davis, 2000; McNish, Gewirtz, & Davis, 1997; Rudy et al., 2002; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). There is evidence that weanling rats sometimes use a hippocampus-independent strategy (Pugh & Rudy, 1996). However, separating the context preexposure from the immediate shock in the CPFE is thought to require the use of a hippocampus-dependent strategy (Rudy et al., 2002). The separation of the context exposure allows the hippocampus-dependent contextual learning to occur in the absence of shock presentation which would normally be required for hippocampus-independent explicitly-cued fear conditioning.

That weanling rats' use a hippocampus-dependent strategy to express fear to a context is also supported by the finding that MK-801 administered during the contextual preexposure impaired the CPFE (Experiment 2). MK-801 impaired the incidental learning that normally occurs during unreinforced exploration of the context similar to previous findings in adult rats following i.c.v. or intrahippocampus infusion of AP5, another NMDA receptor antagonist (Matus-Amat et al., 2007; Stote & Fanselow, 2004). The similar impairment caused by NMDA receptor antagonists in the ability of contextual preexposure to reverse the immediate shock deficit suggests that the cellular mechanisms supporting contextual learning emerge as early as PND 23 in the rat.

Systemic MK-801 injections have been shown to impair a variety of hippocampus-dependent learning and memory tasks during development. For example, MK-801 impairs spatial delayed alternation (Watson, Herbert & Stanton, 2009) and spatial reversal learning (Chadman et al., 2006) at PND 21–30, olfactory discrimination learning at PND 22 (Griesbach, Hu, & Amsel, 1998), delayed patterned-single alternation at PND 16 (Highfield, Nixon, & Amsel, 1996), and acquisition (PND 16–17) and extinction (PND23–24) of cued fear conditioning (Langton, Kim, Nicholas, & Richardson, 2007). Thus, there may be common mechanisms of development that contribute to NMDA receptor involvement in

hippocampus-amygdala interactions. Finally, these experiments demonstrate that this CPFE paradigm is well-suited for examining the ontogenetic time course and neural substrates of contextual learning in developing rodents.

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Figure 1.

The effects of varying context preexposure and the placement-to-shock interval on contextual fear conditioning in PND 23–25 rodents. There was no main effect of preexposure, but there was an effect of varying the placement-to-shock interval. * = significantly different from the preexposed-other rats in the same placement-to-shock interval.

Figure 2.

The effects of MK-801 during preexposure to the context on subsequent contextual fear conditioning to an immediate and delayed shock. * = significantly different from saline treated rats in the same placement-to-shock condition.